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ASSESSING THE PATHOGENICITY OF HALOPHILIC VIBRIO  
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WATER RESOURCES RESEARCH CENTER R 5 JUJIOKA 22 FEB 88

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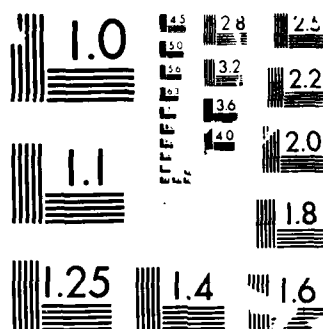
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<p>The health and ill population of dolphins at NOSC, Hawaii, their feed and their environmental sea pens were monitored for normal and for pathogenic bacteria, especially <u>Vibrio</u> spp. The predominant vibrio bacteria recovered from the sea pen water and from the blowholes of healthy dolphins was <u>V. alginolyticus</u>. The recovery of bacteria other than <u>V. alginolyticus</u> from the blowholes of dolphins was indicative that the dolphin's health was being compromised. <u>V. damsela</u>, a known pathogen, was determined to cause wound infections in dolphins. Judicious use of antibiotics can be used to treat these wound infections. Strong evidence was obtained that <u>V. vulnificus</u>, the most virulent species of vibrio caused internal infection and death of a dolphin. Other bacteria implicated in causing diseases and deaths in dolphins are <u>Staphylococcus aureus</u>, <u>Escherichia coli</u>, and <u>Pasteurella multocida</u>.</p>					
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Honolulu, Hawaii 96822

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### TABLE OF CONTENTS

- I. Project Goals
- II. Establishing Relationships with NOSC, Hawaii
- III. Establishing the Blood Serum Collection Program
- IV. Evaluation of Methodology
- V. Concentrations of Vibrio in Hawaii's Marine Water
- VI. Recovery of Vibrio from Crustaceans and Mollusks in Seapens
- VII. Recovery of Vibrio from Dolphin Feed
- VIII. Microbiological Assessment of Dolphins at NOSC
- IX. Recovery of Vibrio from Dolphins Transported to Hawaii
- X. Vibrio Damsela Infections of Wounds in Dolphins
- XI. Vibrio Vulnificus Infection of Dolphin
- XII. Assay for Plasmid from Vibrio Isolates
- XIII. Microbiological Assays of Newly Capture Dolphins
- XIV. Recovery of Staphylococcus Aureus from Dolphins
- XV. Recovery of Pasteurella Multocida from Dolphins
- XVI. Recovery of Escherichia Coli from Dolphins
- XVII. Immunological Response by Dolphins
- XVIII. Summary
- XIX. Presentations and Publications
- XX. Personnel

## **PROJECT FINAL REPORT**

### **I. Project Goals and Objectives**

The goal of this study was to identify the pathogenic microorganisms which infect and cause diseases in marine mammals held in coastal sea pens at Naval Ocean Systems Center (NOSC) located in Kaneohe Bay, Hawaii. Specific objectives of this study are listed as follows:

1. To compare the background concentrations of bacteria, especially Vibrio spp. in sea pen waters as compared to open ocean waters.
2. To identify the vibrio bacteria recovered from sediment, crustaceans and shellfish in the sea pen, as well as fish used to feed dolphins of dolphins at NOSC, Hawaii.
3. To identify the bacteria, especially Vibrio spp. from the blood, feces, blowhole, and skin sites of dolphins kept at NOSC.
4. To identify the bacteria, especially Vibrio spp. from wounds of apparently infected dolphins as well as from internal organs of dolphins who have died. These bacterial isolates were then biotyped (antibiotic sensitivity, presence of plasmids, or phage typed) to determine whether these markers correlate with their pathogenic potential.
5. To collect, date/identify and store blood serum from all the marine mammals and animal handlers at NOSC, Hawaii for future analyses.

### **II. Establishing Working Relationship With NOSC, Hawaii**

The site for this study was the marine mammal pens at NOSC, Hawaii housed within the Kaneohe Marine Corp Air Station. Dr. J. Peter Schroeder, Research Veterinarian, who is responsible for the health of the marine mammals at NOSC, was our authorized contact person there. The objectives, experimental design, and logistics for conducting our study were discussed and approved by Dr. Schroeder. The cooperation extended to us by Dr. Schroeder and his staff throughout this study with regard to obtaining clearance to enter the site, to obtaining samples, to obtaining relevant information and for their scientific and technical assistance were excellent.

### **III. Establishing the Blood Serum Collection Program**

One of the first program which was initiated was the collection of periodic blood serum samples from all the important marine mammals as well as from the human handlers. These serum samples were identified, labeled, and stored for future antibody analyses. These serum samples represent historical records of the infections of marine mammals as well as their human handlers. They are planned for future studies and can be used to verify specific infections in marine mammals and possibly cross infections between marine mammals and human handlers of specific microbial pathogens, such as viruses.

#### IV. Evaluation of Methodology

Most of the published methods to specifically recover the different species of vibrio were evaluated. The membrane filtration method was determined to be suitable for recovering bacteria from water samples while plating directly onto agar media or into broth culture was determined to be the appropriate technique for recovering bacteria from solid samples such as skin. Five experimental media (mVP, TTG, TSAT, MAAC, VV) recently reported in the literature were tested to recover specific species of Vibrio from waters in Hawaii. None of these media were sufficiently selective or differential in recovering specific species of vibrio from the marine waters of Hawaii. Non-vibrio bacteria grew readily on some of these media and these media were not as selective for the vibrio species as reported.

The traditional thiosulfate-citrate-bile salt (TCBS) agar medium was determined to be the best medium to selectively and quantitatively recover the various Vibrio spp. from Hawaii's marine water samples. Thus, TCBS and the more selective TCBS supplemented with 7% NaCl was used in the initial isolation of vibrio bacteria from dolphin and marine water samples. Marine agar was used to recover total marine bacteria, while blood agar was used to recover hemolytic strains of bacteria, including Staphylococcus aureus from dolphins and marine water samples. The mFC or mTEC agar media was used to recover fecal coliforms or E. coli while the mEnt or mE agar were used to recover enterococci bacteria from marine water samples.

The API 20E enterobacteria identification kit was initially used to identify the isolated vibrio bacteria. However, this 26 test system was determined to be inadequate and therefore the more extensive 56 test identification system as described by West and Colwell was used to identify the various species of vibrio. The coagulase test and latex agglutination test were used to identify Staphylococcus aureus isolates. The Kirby Bauer method was used to determine the antibiotic susceptibility of the various bacterial isolates. The Portnoy method was used to recover plasmids from isolates.

#### V. Concentrations of Vibrio in Hawaii's Marine Waters

In temperate waters such as in Chesapeake Bay or in Tokyo Bay, the concentrations of vibrio bacteria increases to high levels during the warmer summer months and virtually disappear during the colder winter seasons when the water temperature drops below 10 C. The results of this study showed that vibrio bacteria can be readily recovered from the coastal waters of Hawaii throughout the year. These results correlate with the fact that Hawaii is located in the tropical zone where the temperature of the coastal marine waters remains 24 +/- 4 C throughout the year.

V. alginolyticus is by far the predominant species of vibrio present in Hawaii's marine waters. However, other vibrio species such as V. cholerae non-01, V. fluvialis, V. parahaemolyticus, V. vulnificus, V. marinus, V. harveyi, V. campbelli, V. nigripulchritudo, V. pelagius, V. splendidus as well as some unspeciated vibrios were also recovered from the environmental waters. The concentrations of vibrio was higher in near

coastal waters as compared to marine water samples obtained greater than one mile offshore which ranged from 52-514 CFU/100 ml. The total vibrio concentrations within and near the seapens ranged from 413 to 9,862 CFU/100 ml. In comparison, the total marine bacteria in these waters ranged from 1,600,000 to 7,800,000 CFU/100 ml. These vibrio isolates were consistently resistant or had intermediate sensitivity to the following antibiotics: penicillin G, ampicillin, carbenicillin, streptomycin, and kanamycin. In contrast, these isolates were sensitive to chloramphenicol, sulfadiazine, trimethoprim/ sulfamethoxazole as well as the vibrio static agent 0/129.

The sanitary quality of marine waters is determined by the concentrations of fecal indicator bacteria such as fecal coliforms. Recreational waters with less than 200 fecal coliforms per 100 ml water sample is considered safe for swimming. Dolphin seapen waters were periodically assayed for fecal indicator bacteria. Results of these analysis revealed low levels (0-20/ 100 ml) of fecal coliforms, indicating that the sanitary quality of the dolphin seapen waters was good.

#### **VI. Recovery of Vibrio from Crustaceans and Mollusks in Seapens**

Vibrio alginolyticus was the predominant (>60%) species of vibrio recovered from water samples obtained in the dolphin seapens. This same species of vibrio was also the predominant vibrio recovered from silt and coral samples obtained from the bottom of these seapens which are approximately 12 feet deep. Crabs were the most numerous benthic crustaceans. Three species of benthic crabs were sampled and also yielded V. alginolyticus as the predominating bacterial species. Fish and coelenterates which were present in these seapens were not analyzed.

Isognomon, a marine mollusk indigenous to the shallow coastal waters in Hawaii and known to filter feed were placed in the dolphin seapen. These mollusks were harvested after more than a week of residence and their entire tissues analyzed for vibrio bacteria. The predominant vibrio recovered from Isognomon was V. parahaemolyticus, a species of vibrio which was only rarely recovered from water samples taken from the seapen. These results indicate that these mollusk can preferentially recover a specific species of vibrio from the surrounding water.

#### **VII. Recovery of Vibrio from Dolphin Feed**

Dolphin feed was frozen smelt and mackerel fish which were shipped to Hawaii from the mainland (Oregon, Great Lakes or California). Vibrio fluvialis (I) was recovered from the intestinal tract and surface of these feed fish at relatively low concentrations.

#### **VIII. Microbiological Assessment of Dolphins at NOSC**

Since microorganisms cause disease and since the seapens water contain many kinds of bacteria, samples of blood, feces, skin, and blowholes of dolphins were assayed for bacteria, especially Vibrio spp.

Samples of blood from dolphins were consistently negative for vibrio bacteria. Swabs of the unwashed skin and blowhole of dolphins routinely

resulted in recovery of V. alginolyticus. This was not unexpected since these sites were constantly bathed in seawater in which this same species of vibrio predominated. Various species of bacteria including Proteus mirabilis, Edwardsiella tarda, Pseudomonas putida, Flavobacterium odoratum, as well Vibrio spp. were recovered from fecal samples of dolphins. However, sucrose negative V. parahaemolyticus was present in higher concentrations than the sucrose positive V. alginolyticus.

#### **IX. Recovery of Vibrio from Dolphins Transported to Hawaii**

In August of 1984, ten dolphins were transported from Gulfport, Mississippi to NOSC, Hawaii. These dolphins had been captured off Gulfport, Mississippi within the previous 30 days. Upon arrival in Hawaii, blood samples as well as blowhole samples were immediately taken and analyzed for vibrio bacteria. All blood samples were negative for vibrio and other bacteria. V. parahaemolyticus was the predominant vibrio recovered from the blowholes of these dolphins. These results contrasted with the routine recovery of V. alginolyticus from the blowholes of dolphins housed at NOSC, Hawaii. However, after being housed in NOSC, Hawaii for two to eight weeks, the predominant vibrio recovered from the blowholes of the dolphins transported to Hawaii was V. alginolyticus. Two interpretations can be drawn from these results. First, that the predominant vibrio in Hawaiian waters is V. alginolyticus whereas the predominant vibrio in the gulf of Mexico is V. parahaemolyticus. Second, transporting these dolphins result in stressing these animals which allows V. parahaemolyticus to become established in the blowhole area.

#### **X. Vibrio Damsela Infections of Wounds in Dolphins**

Some of the bottlenose dolphins (Tursiops truncatus) kept in the coastal waters at NOSC, Hawaii developed slow healing wounds which clinically appeared to be infected. These animals were treated with Tribrissen antibiotics (trimethoprim/sulfadiazine) which usually resulted in cure. The predominant vibrio recovered from the sea pen water and from the open ocean was V. alginolyticus while the predominant vibrio recovered from the healthy skin of dolphins was V. marinus. Both of these vibrio isolates were sensitive to the antibiotics (Tribrissen) used to treat the dolphins. The predominant vibrio recovered from the wounds of dolphins was V. damsela which is a known pathogen of fish and man. This vibrio which was specifically recovered from the wounds of dolphins was shown to be developing resistance to trimethoprim or sulfadiazine. Based on these results, it was concluded that V. damsela was infecting (multiplying) at the wound site and was believed to be the primary bacteria causing wound infections in dolphins.

#### **XI. Vibrio Vulnificus Infection of Dolphin**

A dolphin housed at NOSC, Hawaii was under observation for a long history of chronic illness, including inappetence, weight loss, and gastric ulcers. As a result of very lethargic behavior and rapid, shallow respiration, this dolphin was lifted out of the water for further examination. The dolphin died suddenly in the stretcher while undergoing examination. This animal was immediately taken to the Veterinary Facility



at NOSC where post-mortem necropsy was performed. During this autopsy, internal organs were also sampled for presence of bacteria.

Vibrio vulnificus was consistently recovered from various internal organs of the expired dolphin, often in pure culture. This bacteria was recovered on the three bacteriological media (blood agar, marine agar, TCBS agar) used indicating the pervasiveness of this bacteria in the blood, spleen, kidney, and stomach. In comparison, no bacteria was recovered from internal organs of another dolphin which had expired under similar conditions. Based on these results and the known virulence of V. vulnificus, we conclude that septicemia resulting from V. vulnificus infection of this dolphin was instrumental in causing the death of this animal.

#### XII. Assay for Plasmids from Vibrio Isolates

A total of 24 vibrio isolates recovered from wound infections, from healthy skin and from environmental waters were assayed for presence of plasmids by the following three methods: 1. Takahashi, 2. Kado and Liu, 3. Portnoy. No plasmids were detected from these 24 vibrio isolates using these three methods. Plasmids were recovered from an Erwinia bacteria and from a known V. parahaemolyticus indicating that the methods were working. More recently, a single large plasmid (100-150 megadalton) was detected from the V. vulnificus isolated from the internal organs of a recently expired dolphin.

#### XIII. Microbiological Assays of Newly Captured Dolphins

Wild dolphins captured near the Hawaiian islands and brought back to the NOSC, Hawaii facilities were monitored for bacteria immediately after capture and for several weeks after capture. The predominant bacterial flora of the blowholes from these dolphins was Pseudomonas spp. However, after living in the NOSC facilities for a few weeks, the predominant bacteria recovered from the blowholes of these dolphins was determined to be V. alginolyticus. A correlation between V. alginolyticus recovery from the blowholes of dolphins housed in NOSC, Hawaii and the general health of dolphins was observed. V. alginolyticus was the predominant bacteria recovered from blowholes of healthy dolphins at NOSC. Various other types of bacteria such as Pseudomonas spp., Pasteurella spp., V. parahaemolyticus, Staphylococcus aureus, and Escherichia coli were recovered from blowhole samples of dolphins which appeared to be ill by their behavior or by their blood chemistry results. These results suggest that monitoring the bacterial flora of the blowholes of dolphins at NOSC, Hawaii may be a good indicator of the health and well being of dolphins.

#### XIV. Recovery of Staphylococcus Aureus from Dolphins

Two of the dolphins captured from Hawaiian waters died, one in 9 days and one after 46 days in captivity. Immediately after expiring, these two dolphins were autopsied at the NOSC, Hawaii facilities. Staphylococcus aureus was the only bacteria recovered from several of the internal organs of these two expired dolphins. Significantly, the S. aureus recovered from the two expired dolphins was coagulase positive

and resistant to penicillin. These results indicated that Staphylococcus aureus infections leading to septicemia was instrumental in the deaths of these two dolphins.

As a result of recovering S. aureus from these two dead dolphins, monitoring for S. aureus was initiated to determine the source of Staphylococcus aureus. The seapen water was considered the most likely source of infection for dolphins and was therefore analyzed for S. aureus. Presumptive colonies of S. aureus ranging in concentrations from 7 to 16 CFU/100 ml were recovered from seapen water samples. Human dolphin handlers were also considered a likely source of infection for dolphins and were analyzed for S. aureus. One of the primary dolphin handlers was found to be a nasal carrier of S. aureus. The S. aureus isolated from the seapen water and from the human handler was similar to the isolates from the dead dolphins in their resistance to penicillin. However, phage typing analysis of the S. aureus isolates revealed differences in phage sensitivities suggesting that the source of the S. aureus from the dolphin, from the seapen water and from the human handler was not the same. Thus, the source and vector by which S. aureus infected dolphins has not been determined. However, the evidence indicates that S. aureus infections of dolphins may lead to death.

#### XV. Recovery of Pasteurella Multocida from Dolphins

Pasteurella multocida is a known pathogen of animals, birds, and even humans. However, this bacteria has also been recovered from apparently healthy animals and humans. Why some strains of P. multocida are virulent while others are not remains unclear. P. multocida was recovered from the blowholes of some dolphins, primarily dolphins which had recently arrived in NOSC, Hawaii. The significance of recovering P. multocida from dolphins has yet to be determined but must be considered a probable disease causing infection in dolphins.

#### XVI. Recovery of Escherichia Coli from Dolphins

Escherichia coli is usually not pathogenic for humans and animals. However, there are some strains of E. coli which produce a toxin and thereby becomes pathogenic. Since E. coli is an enteric bacteria, its recovery from the blowholes of some dolphins was unexpected. Significantly, E. coli has been recovered as one of the predominant bacteria from the internal organs of two recently expired dolphin. These results indicate that E. coli may be capable of establishing a pathogenic infection in dolphins.

#### XVII. Immunological Response by Dolphins

Mammals usually respond to microbial infections by producing antibodies to these microorganisms. Much is known of the immune response of humans and some animals to microbial infections. However, the immune response of dolphins to microbial infections has not been well characterized. This study indicated that several genera of bacteria (Vibrio, Pasteurella, Staphylococcus, Escherichia) infected and were pathogenic for dolphins.

A preliminary study was initiated to determine whether dolphins were producing antibodies to these suspected bacterial infections. Dolphin sera were used as source of antibodies. The isolated bacteria were used as sources of antigen for three tests (Microagglutination, hemagglutination, Ouchterlony). Evidence was obtained that the dolphins at NOSC, Hawaii are producing antibodies to Vibrio and Pasteurella spp.

#### XVIII. Summary

The healthy and ill population of dolphins at NOSC, Hawaii, their feed and their environmental sea pens were monitored for normal and for pathogenic bacteria, especially Vibrio spp. The predominant vibrio bacteria recovered from the sea pen water and from the blowholes of healthy dolphins was V. alginolyticus. The recovery of bacteria other than V. alginolyticus from the blowholes of dolphins was indicative that the dolphin's health was being compromised. V. damsela, a known pathogen, was determined to cause wound infections in dolphins. Judicious use of antibiotics can be used to treat these wound infections. Strong evidence was obtained that V. vulnificus, the most virulent species of vibrio caused internal infection and death of a dolphin. Other bacteria implicated in causing diseases and deaths in dolphins are Staphylococcus aureus, Escherichia coli, and Pasteurella multocida.

#### XIX. Presentations and Publications

1. Greco, S., R. Fujioka, M. Cates, and P. Schroeder. 1985. Assessing the concentrations and significance of vibrio bacteria in pens used to raise marine mammals. Presented at the Spring Conference of the Hawaii Branch of American Society for Microbiology. Chaminade University. April 20, 1985.

2. Greco, S., R. Fujioka, M. Cates and P. Schroeder. 1985. Assessing the concentrations and significance of vibrio bacteria in pens used to raise marine mammals. Proceedings of the 16th Annual Conference of the International Association for Aquatic Animal Medicine. Vol. 1-2, pp. 139-145.

3. Fujioka, R.S., S.B. Greco, M.B. Cates and J.P. Schroeder. 1986. Vibrio spp. associated with wounds from bottlenose dolphins housed in sea pens. Abstract of the 1986 Annual Meeting of the American Society for Microbiology. March 23-28, 1986, Washington, D.C. p. 94.

4. Greco, S.B., R.S. Fujioka, and J.P. Schroeder. 1986. Patterns of antibiotic resistance in Vibrio spp isolated from cold blooded animals, marine mammals, and environmental sources. Proceedings of the 17th Annual Conference of the International Association for Aquatic Animal Medicine. Vol. 1-3, pp 136-140. May 4-7, 1986, Biloxi, Mississippi.

5. Fujioka, R., S. Greco, M. Cates, and J. P. Schroeder. 1987. Isolation of Vibrio vulnificus from internal organs of a suddenly expired atlantic bottlenose dolphin. Abstract of the 87th Annual Meeting of the American Society for Microbiology. p. 352. Atlanta, Ga., March 1-6, 1987.

6. Fujioka, R., S. Greco, M. Cates and J. P. Schroeder. 1987. Presented at the 18th Annual Conference of the International Association for Aquatic Animal Medicine. Monterey, California, May 10-13, 1987.

7. Fujioka, R.S., S.B. Greco, M.B. Cates, and J. P. Schroeder. 1988. Vibrio damsela from wounds in bottlenose dolphins, Tursiops truncatus. Accepted for publication in Diseases of Aquatic Organisms.

8. Palmer, C.J., R.S. Fujioka, and J.P. Schroeder. 1988. Staphylococcus aureus infections in the Pacific Bottlenose dolphin. Accepted for presentation at the 1988 Annual Meeting of the American Society for Microbiology, May 8-13, 1988 at Miami Beach, Florida.

#### **XX. Personnel Supported**

1. Stephen Greco. Ph.D. candidate in Microbiology.
2. Carol Palmer. Ph. D. candidate in Microbiology.
3. Myron Honda. MS. candidate in Microbiology.

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